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Activation of the JNK/p38 Pathway Occurs in Diseases Characterized by Tau Protein Pathology and Is Related to Tau Phosphorylation But Not to Apoptosis

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Abstract. JNK and p38, two members of the MAP kinase family, are strongly induced by various stresses including oxidative stress and have been involved in regulation of apoptosis. As both kinases phosphorylate tau protein in vitro, we have investigated their immunohistochemical localization in a group of neurodegenerative diseases characterized by intracellular deposits of hyperphosphorylated tau. Cases included Alzheimer disease, Pick disease, progressive supranuclear palsy, corticobasal degeneration, Gerstmann-Sträussler-Scheinker disease-Indiana kindred, and frontotemporal dementia with parkinsonism linked to chromosome 17. In all tissue samples, strong immunoreactivity for both MAP kinases was found in the same neuronal or glial cells that contained tau-positive deposits. By double immunohistochemistry, JNK and p38 colocalized with tau in the inclusions. Analysis of apoptosis-related changes (DNA fragmentation, activated caspase-3) showed that the expression of JNK and p38 was unrelated to activation of an apoptotic cascade. Our data indicate that phospho-JNK and phospho-p38 are associated with hyperphosphorylated tau in a variety of abnormal tau inclusions, suggesting that these kinases may play a role in the development of degenerative diseases with tau pathology.

Key Words: Apoptosis; JNK; MAP kinase; Phosphorylation; p38; Tau; Tauopathy.

INTRODUCTION

A hallmark of several neurodegenerative diseases is the intracellular accumulation of insoluble protein aggregates that interfere with proper cell functioning and are likely to be responsible for disease progression (1, 2). Inclusions made of hyperphosphorylated tau protein occur in a variety of degenerative conditions (3), which include most cases of dementing illness among elderly humans (4). Major diseases with tau pathology are Alzheimer disease (AD), Pick disease (PD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), a tauopathy due to mutations in the *TAU* gene, and Gerstmann-Sträussler-Scheinker (GSS) disease caused by a mutation at codons 198 and 217 in the prion protein gene (3, 5–7).

Tau is a microtubule-associated protein that is found in neurons (8) and oligodendrocytes (9) and exists in the adult brain in 6 different isoforms (10). In all tau-related diseases, the protein is abnormally phosphorylated relative to that from normal brain and forms intraneuronal and/or glial filamentous inclusions. The appearance and distribution of the inclusions, the biochemical composition of tau isoforms and their phosphorylation state vary in the different disorders (3, 5, 11–15). Data from various model systems suggest that phosphorylation at abnormal sites is a critical step in the process of tau self-aggregation and insolubility (4, 13); however, the identification of the kinase(s) involved has not been accomplished.

Among the various kinases that are able to phosphorylate tau in vitro (13), c-Jun N-terminal kinase (JNK) and p38 kinase, 2 members of the mitogen-activated protein (MAP) family of kinases, are particularly interesting candidates (16–19). In vitro, both kinases phosphorylate tau at sites that are only phosphorylated in AD tau but not in normal tau (16, 18, 19). Moreover, JNK and p38 are activated by various forms of stress (20, 21), including oxidative stress, which has been implicated in the pathogenesis of AD (22), and are also implicated in apoptosis (20, 21, 23, 24). A few recent reports have linked JNK and p38 activation to abnormal tau phosphorylation and neurodegeneration in AD brain (17, 22, 25, 26). In contrast, very little data exist on the kinases involved in diseases other than AD. Activation of ERKs, another member of the MAP kinase family that is expressed in AD brain (27, 28), has been recently shown to occur in various tau-related diseases (29).

In this report we have investigated whether p38 and JNK are involved in abnormal tau phosphorylation and/or in other activities relevant for disease pathogenesis and progression (e.g. apoptosis) in a series of cases of AD and other diseases with tau pathology.

MATERIALS AND METHODS

The immunohistochemical expression of JNK and p38 MAP kinases was investigated in a series of neuropathologically confirmed cases of (a) sporadic AD (n = 5); (b) familial AD (FAD)

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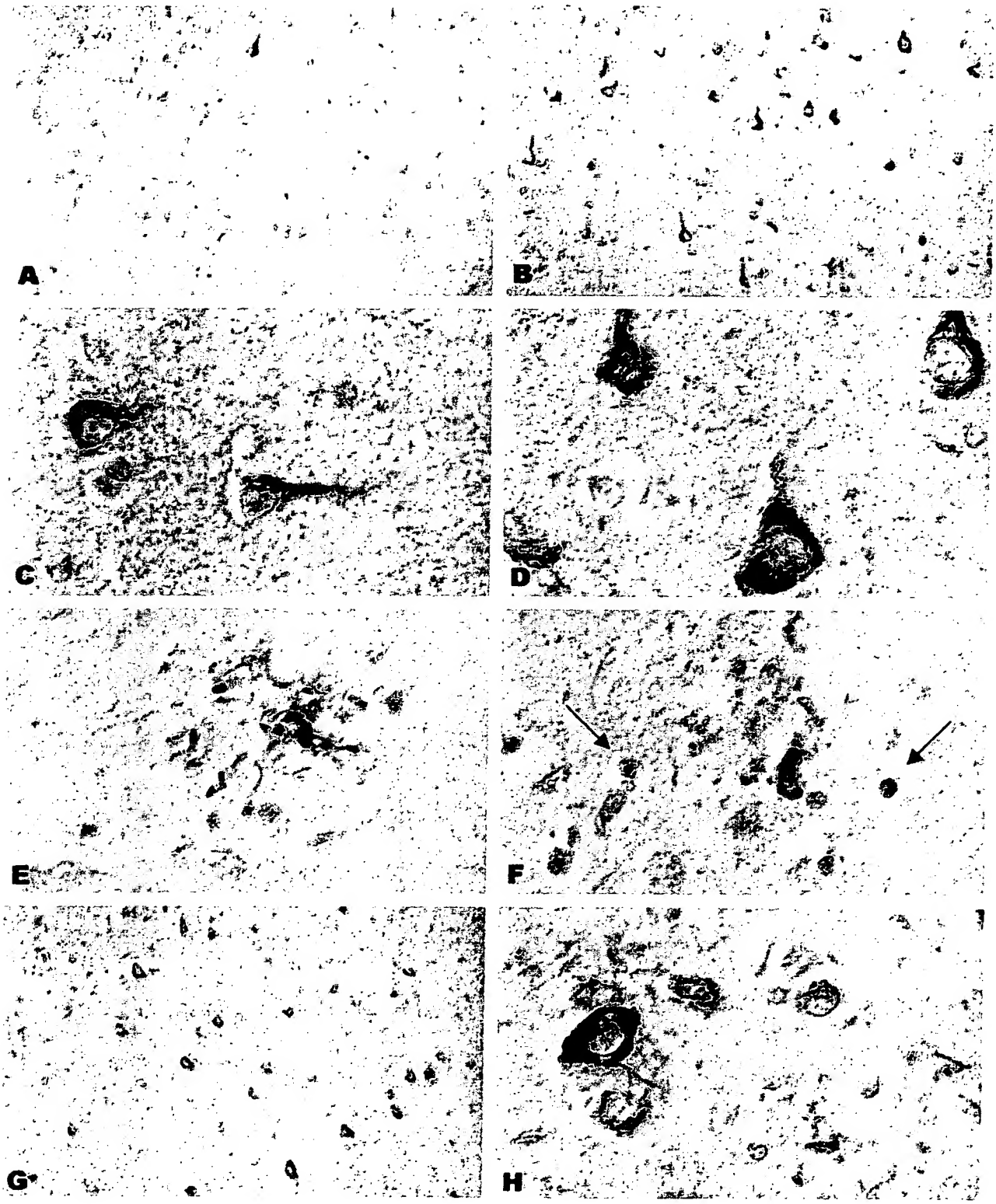

Fig. 1. p38 immunoreactivity in control (a) and AD (b) brain. Only isolated immunoreactive neurons are found in control tissue, whereas large numbers of neurons are labeled in AD brain. At the cellular level, however, the labeling intensity is identical. At high power view, both p38 (c) and JNK (d) antibodies decorate intraneuronal neurofibrillary tangles. Dystrophic neurites surrounding amyloid deposits are prominently stained by both anti-JNK (e) and anti-p38 (f) antibodies. Occasional glial nuclei are also labeled (f, arrow). g, h: A similar pattern of immunoreactivity is found in neurons and neurites of plaques in GSS-Indiana kindred brain (Fig. 1g, h). Magnifications: a, b, g, ×200; c–f, h, ×1,000.

with APP V717F mutation (30) (n = 3); (c) FTDP-17 with 3'Ex10+3 mutation (31) (n = 4); (d) FTDP-17 with P301L mutation (32) (n = 1); (e) FTDP-17 with N279K mutation (33) (n = 1); (f) FTDP-17 with G389R mutation (34) (n = 1); (g) PD (n = 2); (h) CBD (n = 2); (i) PSP (n = 2); (j) GSS with F198S mutation (Indiana kindred) (n = 2); and (k) age-matched controls (n = 5). Available clinical data are listed in the Table. Autopsy samples from representative cortical and subcortical areas affected by tau pathology (Table) were fixed in formalin, dehydrated, and embedded in paraffin. Sections were cut at 6 μm and mounted on Superfrost® slides (Portsmouth, NH).

After deparaffinization and inhibition of endogenous peroxidase activity, sections were immunostained with polyclonal rabbit antibodies to phosphorylated (i.e. activated) p38 (diluted 1/100, New England Biolabs, Beverly, MA) and to phosphorylated JNK (1/100, Santa Cruz Biotechnology, Santa Cruz, CA). For p38, specificity of the immune reaction was tested by preadsorbing the antibody with the immunizing peptide (kindly provided by Dr. Andreas Nelsbach, New England Biolabs). Deposits of hyperphosphorylated tau were revealed with a monoclonal antibody (AT-8, 1/300, Innogenetics, Ghent, Belgium). AT-8 specifically recognizes a phosphorylated epitope of tau that is present in pathologic tau of AD as well as in other tau-related disorders. All immune reactions were visualized with a standard streptABC technique, using diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) as substrate. For double labeling studies, sections were first immunostained with AT-8, followed by a fluorescent Cy3-conjugated secondary antibody, and the tau deposits were photographed. After ethanol decoloration and elution of the primary antibody with HCl, sections were immunostained with the polyclonal antibody to JNK or p38 and the reaction was revealed using DAB as substrate.

To investigate the occurrence of apoptotic changes, we used a polyclonal antibody that selectively recognizes activated caspase-3, the main downstream effector protease of apoptosis (CM1, 1/2,000, IDUN Pharmaceuticals, Inc., La Jolla, CA). To detect nuclear DNA fragmentation, a hallmark of apoptosis, an in situ-end-labeling (ISEL) technique was used. Pretreatment of sections with proteinase K (a prerequisite for successful in situ staining of fragmented DNA) was performed at low concentration of the enzyme (0.5–5 μg/ml for 15 min at room temperature) in order to avoid nonspecific staining of nuclei, a frequent event when using autopsy material (35). DNA labeling was performed by incubating sections with terminal deoxynucleotidyl transferase (20 U/100 μl, Roche Diagnostics, Indianapolis, IN) and fluorescein-11-dUTP (1 nmol/100 μl, Roche Diagnostics) for 2 h at 37°C. The reaction was revealed with peroxidase-conjugated anti-fluorescein antibody (Roche Diagnostics) using diaminobenzidine (DAB) as chromogen (36). For both caspase-3 and ISEL assays, sections from a case of infantile pontosubicular necrosis (PSN) were included as positive control for neuronal apoptosis (37).

RESULTS

In sections of frontal and temporal lobe from control subjects, strong immunoreactivity to both p38 and JNK was occasionally found in the cytoplasm of single pyramidal neurons bearing neurofibrillary tangles (NFTs) (Fig. 1a). In adjacent sections stained with AT-8, neurons containing hyperphosphorylated tau were found, with similar morphology and frequency as those seen with JNK and p38 antibodies (not shown). All other cortical and subcortical neurons and glial cells were totally devoid of JNK and p38 immunoreactivity.

In pathological tissue samples, immunolabeling for p38 and JNK occurred in a far greater number of neuronal and/or glial cells than in control cases. As in controls, the regional pattern of distribution of p38 and JNK closely matched that seen for hyperphosphorylated tau in adjacent sections. In AD, antibodies to both MAP kinases strongly decorated the cytoplasm of most cortical neurons and their processes, without significant differences among the 2 kinases in the pattern and frequency of immunolabeling (Fig. 1b). At high power view, the labeling was localized on NFTs located in the neuronal perikarya (Fig. 1c, d), and only rarely in extracellular NFTs. In addition, dystrophic neurites surrounding amyloid deposits were prominently stained (Fig. 1e). Occasional labeling was also noted in the nuclei of glial cells located in the vicinity of amyloid plaques (Fig. 1f). A very similar pattern of labeling in neurons and neurites of plaques occurred in the GSS case (Fig. 1g, h). In the FTDP-17 cases, p38- and JNK-positive structures were localized both in cortical neurons and in oligodendrocytes of the subcortical white matter (Fig. 2a, b). In PD, prominent immunoreactivity was found in neurons with Pick bodies as well as in scattered oligodendrocytes and astrocytes (Fig. 2c–e). In the CBD cases, immunoreactivity for JNK and p38 was mainly found in subpopulations of neurons in the basal ganglia, and quite prominently in most white matter oligodendrocytes with coiled bodies (Fig. 2g, h). On the contrary, cortical achromatic neurons were unlabeled (not shown). In addition, diffuse immune reactivity was present in the cortical astrocytic plaques (Fig. 2f). In the PSP cases, midbrain neurons showed prominent and diffuse immunostaining in their perikarya. Oligodendroglial cytoplasms and coiled bodies were also strongly stained (Fig. 3a, b).

To further characterize the pathology of neuronal and glial elements expressing p38 and JNK, double-labeling studies were carried out using AT-8 monoclonal antibody. Double immunolabeling demonstrated a close correlation in the cellular distribution between p38/JNK and abnormally phosphorylated tau in all major histopathological lesions of AD and of the other diseases with tau pathology (Fig. 3c–f). MAP kinases and tau colocalized in all cytoplasmic aggregates independently of their neuronal/glial origin or morphological profile. Any difference found in the distribution of JNK/p38 positive structures between the various brain areas, and in the individual patients studied, strongly reflected the heterogeneity of tau localization. In summary, we observed a complete overlap in the distribution of p38/JNK and tau cellular immunoreactivity in each tau-related disease investigated.

TABLE
Clinical Data of Cases Selected for the Study

Case #	Disease	Mutation	Age at death	PMI (hours)	Area studied
1	AD		77	12	FC, TC
2	AD		85	17	FC, TC
3	AD		83	NA	FC, TC
4	AD		78	15	FC, TC
5	AD		82	18	FC, TC
6	FAD	APP V717F	48	2	TC
7	FAD	APP V717F	53	19	TC
8	FAD	APP V717F	46	4	TC
9	FTDP-17	Tau 3'E × 10 + 3	58	5	TC
10	FTDP-17	Tau 3'E × 10 + 3	54	12	TC
11	FTDP-17	Tau 3'E × 10 + 3	61	1	TC
12	FTDP-17	Tau 3'E × 10 + 3	64	3	TC
13	FTDP-17	Tau P301L	55	NA	TC
14	FTDP-17	Tau N279K	53	NA	FC
15	FTDP-17	Tau G389R	43	NA	TC
16	PD		78	19	FC, TC
17	PD		82	14	FC, TC
18	CBD		52	NA	TC, BG
19	CBD		59	NA	TC, BG
20	PSP		73	15	MB
21	PSP		65	18	MB
22	GSS	PrP F198S	61	9	TC
23	NC		77	19	FC, TC
24	NC		61	13	FC, TC
25	NC		59	18	FC, TC
26	NC		82	22	FC, TC
27	NC		68	15	FC, TC

Abbreviations: AD = Alzheimer disease; FAD = familial AD; FTDP-17 = fronto-temporal dementia with parkinsonism linked to chromosome 17; PD = Pick disease; CBD = corticobasal degeneration; PSP = progressive supranuclear palsy; GSS = Gerstmann-Straussler-Scheinker disease; NC = normal control.; NA = not available; FC = frontal cortex; TC = temporal cortex; BG = basal ganglia; MB = midbrain.

To analyze whether p38 and JNK activation was related to apoptosis, an ISEL assay was applied to sections adjacent to those immunostained for the MAP kinases. In line with previous observations (29, 35, 38), clearcut ISEL-positive nuclei with apoptotic shape were never identified in any disease with tau pathology, including AD. With increasing proteinase K concentrations, diffuse nuclear staining occurred in most glial and neuronal nuclei of all cases, without any predilection for the areas containing JNK- and p38-positive elements (not shown). To avoid the artefactual staining of ISEL and to assess more precisely whether an apoptotic pathway was activated, we studied the immunohistochemical distribution of the cleaved (activated) form of caspase-3. Strong caspase-3 immune reactivity occurred in apoptotic cells in the PSN case used as control (Fig. 3g). In sections of most tau-related diseases, occasional (1–2/section) caspase-3-immunopositive elements were found. These cells had a condensed nucleus and cytoplasm, indicating an apoptotic nature, but were no longer identifiable as neurons or glia. In particular, caspase-3 immunolabeling was never found in tangle-bearing neurons such as those immunoreactive for tau, JNK, or p38. On the other hand, 2 caspase-3 immunopositive apoptotic cells/section were also found in 2/5 control cases (Fig. 3h). The latter finding suggested that the occasional caspase-3 immunolabeling seen in the tau disorders was not due to the tau pathology, but most likely represented a consequence of disease-unrelated conditions, e.g. an agonal status.

DISCUSSION

Filamentous tau pathology is central to AD as well as to a number of other unrelated dementing disorders (3). In AD, the abnormal phosphorylation of tau, compared to tau from normal adult brain, is thought to be responsible for the detachment of tau from microtubules and its subsequent aggregation into paired helical filaments (4, 11, 13, 39). Interestingly, hyperphosphorylation of tau occurs not only in AD, but also in most diseases with tau pathology (5, 12), suggesting that hyperphosphorylation of tau may be a common event associated with tau aggregation in all these disorders. While several in vitro and in vivo data have identified a number of potentially relevant kinases in AD, only ERKs have been consistently shown to be activated, and possibly linked to tau

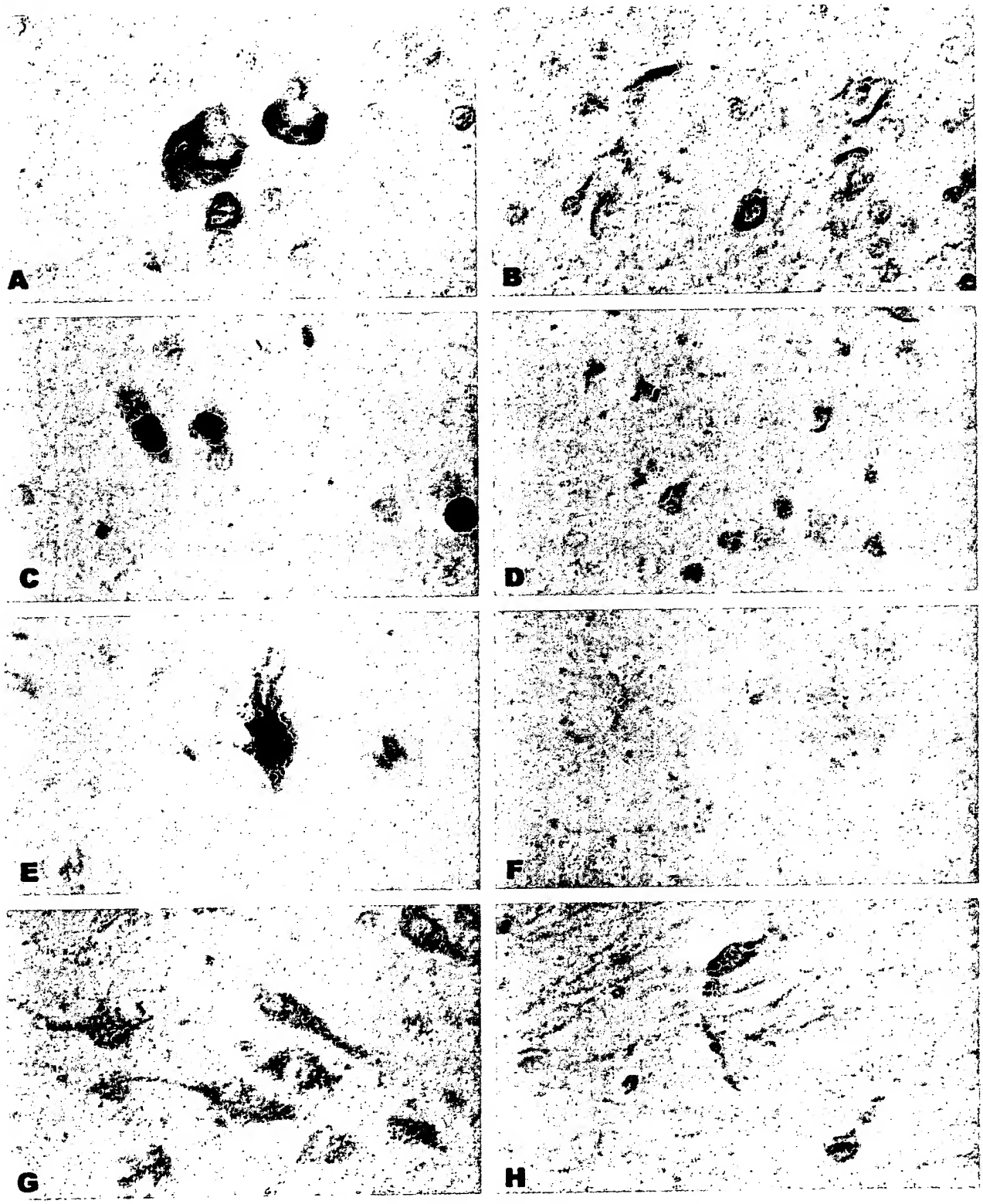

Fig. 2. p38 immunoreactivity in neurons (a) and oligodendrocytes (b) in FTDP-17 brain. c–e: PD brain: antibodies to JNK label Pick bodies of neurons (c) as well as oligodendrocytes (d) and occasional astrocytes (e). f–h: CBD brain: p38 immunoreactivity can be found in astrocytic plaques (f), neurons (g) and oligodendrocytes (h). Magnifications: a–e, g, h, ×1,000; f, ×400.

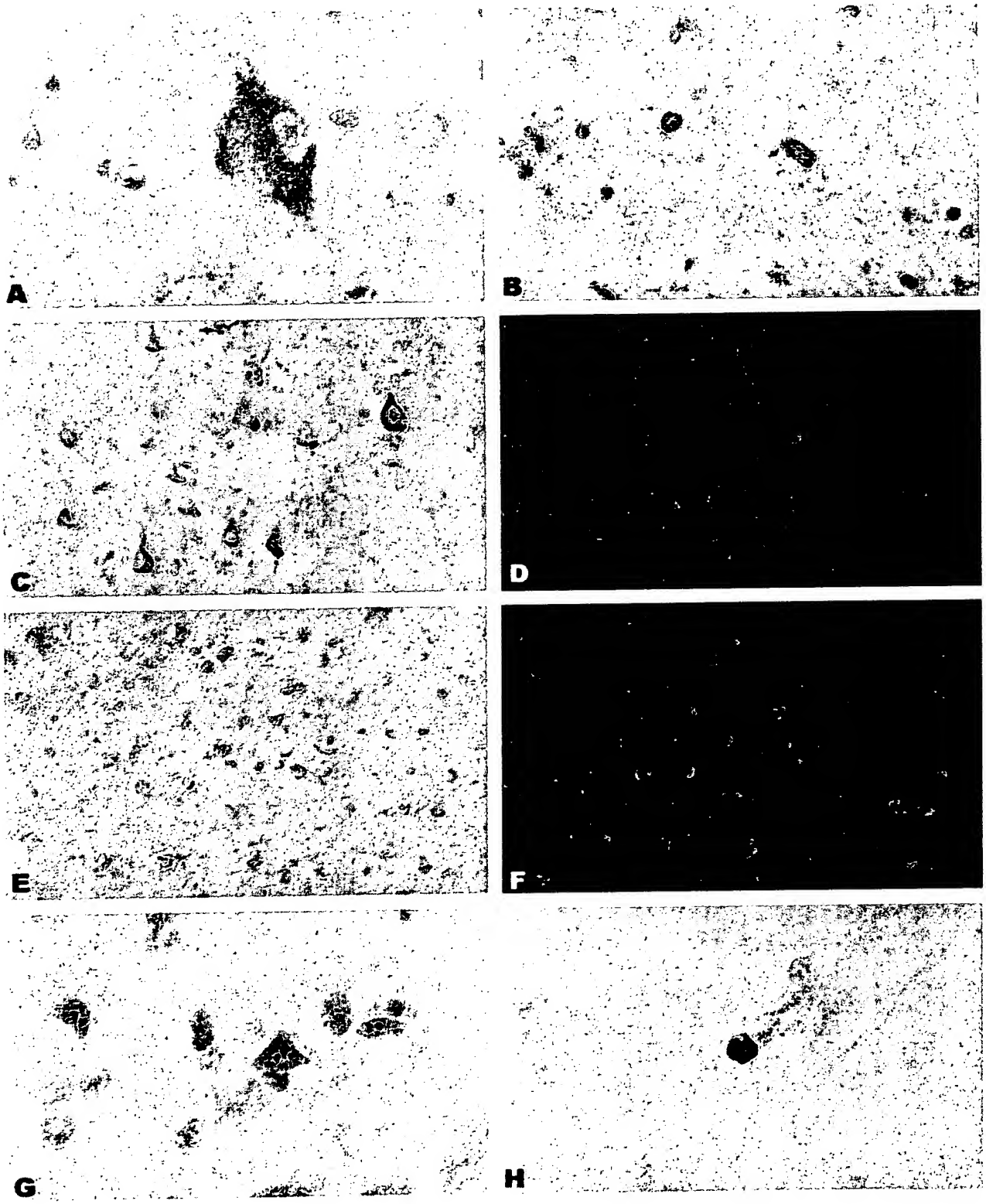

Fig. 3. a, b: JNK immunoreactivity of neurons (a) and oligodendrocytes (b) in PSP. c–f: Double immunostaining in AD (c, d) and FTDP-17 (e, f) shows colocalization of p38 (c, e) and abnormally phosphorylated tau (d, f). g: Antibodies to activated caspase-3 label the cytoplasm of apoptotic neurons in the pons of a case of pontosubicular necrosis. h: A caspase-3 immunoreactive cell is seen in a section from control brain. Similar cells may be occasionally found in tissue sections from all cases with tau pathology. Magnifications: a, b, g, h, ×1,000; c–f, ×400.

phosphorylation, in tau-related diseases other than AD (29).

In this report, we show that JNK and p38 MAP kinases are activated in AD brain. Furthermore, we provide evidence that activation of JNK and p38 also occurs in all major diseases with tau pathology. In these diseases, our analysis revealed a striking overlapping between the immunostaining pattern of hyperphosphorylated tau and that of JNK and p38, by showing that (a) the same neuronal and glial cells that express tau are also JNK and p38-immunoreactive, and (b) antibodies to JNK and p38 decorate all cytoplasmic deposits of tau. Altogether, these data indicate that JNK and p38 colocalize with the tau insoluble aggregates, and strongly argue that the 2 kinases might be involved in tau phosphorylation in any disease characterized by accumulation of hyperphosphorylated tau.

A comparison between our findings and the recently reported data on ERK expression in diseases with tau pathology (29) shows some notable differences between these 2 classes of MAP kinases. In AD brain, JNK and p38 were prominently expressed in all NFTs, including those of dystrophic neurites around senile plaques. In contrast, ERKs were present in neurons with moderate amounts of NFTs but not in neurons with dense NFTs and in dystrophic neurites. Likewise, in tau-related diseases other than AD, JNK and p38 constantly co-localized with hyperphosphorylated tau in all neuronal and glial inclusions, whereas co-localization of ERKs and tau was only present in a subset of cells (29). The significance of these differences is presently unclear, but might suggest either that activation of ERKs precedes that of JNK and p38 in a hypothetical kinase cascade (13), or that ERKs are recruited less constantly than JNK and p38.

What activates JNK and p38 kinases in tau-related diseases? Whereas ERKs respond mainly to mitogenic stimuli, JNKs and p38 are potently activated by deleterious stimuli such as trophic factor deprivation, ischemia, hypoxia, free radicals, and activation of death domain receptors (20, 21), all of which might be important events in a neurodegenerative setting. A role for free radicals in the activation of the JNK/p38 pathway in AD has indeed been hypothesized (22, 26). Some deeper insight in the possible molecular determinants of JNK activation in AD has been recently provided (25). Accordingly, JNK activation was observed in neurons showing intracellular amyloid ß (Aß) immunoreactivity in both mutant PS1 transgenic mice and AD patients (25). Furthermore, treatment of primary cortical neurons with Aß peptide induced JNK activation and cell death, suggesting that intracellular Aß accumulation might trigger JNK activation leading to neuronal death (25). While these data may help to understand the activation of JNK/p38 in AD, the mechanism that activates this pathway in diseases other than AD remains to be determined.

What are the consequences of JNK/p38 activation in these diseases? Although the present data suggest that one likely consequence is the phosphorylation of tau, other effects could be taken into account. JNK and p38 activation has been linked to induction of apoptosis in several experimental paradigms (20, 21, 23, 24). The proapoptotic activity of JNK is likely due to its phosphorylating activity, causing both activation of c-Jun and p53 and inhibition of Bcl-2 (21). As for p38, it is supposed to act synergistically with JNK, but the cellular substrates through which it may induce apoptosis have not been yet identified (21). On the other hand, examples of JNK and p38 involvement in possible neuroprotective rather than neurodegenerative functions have also been provided, suggesting that the actual roles of the 2 MAP kinases are highly cell- and context-dependent (20, 21, 40). The negativity of ISEL and caspase-3 assays in the tau diseases considered is in line with previous observations made in AD brain (29, 35, 38, 41), and suggests that JNK and p38 are not involved in triggering or regulating an apoptotic cascade in diseases with tau pathology, including AD.

In conclusion, our data suggest that JNK and p38 MAP kinases are associated with tau phosphorylation in degenerative conditions characterized by intracellular tau deposits. Since phosphorylation is a likely early step in the formation of insoluble tau aggregates (4) and increases tau resistance to proteolysis (13), activation of the JNK/p38 pathway might contribute to cell dysfunction and death by promoting a degenerative process (i.e. aberrant protein aggregation and accumulation) alternative to apoptosis. In this respect, recent findings have shown that JNK may be activated by accumulation of unfolded damaged proteins, and, conversely, that activation of JNK itself may stimulate the formation of inclusion bodies (2). Further studies on transgenic mice overexpressing mutant tau should allow a better understanding of the role of the JNK/p38 pathway in tau pathology.

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